THE EFFECTS OF CONDITIONS OF HOMOGENIZATION ON THE TYPES OF NUCLEI ISOLATED FROM RAT LIVER

A Thesis

Presented to

The School of Graduate Studies

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

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September 1971

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INTRODUCTION AND REVIEW OF THE LITERATURE

The widespread use of techniques of homogenization and centrifugation for isolating rat liver nuclei makes the dependability of these techniques important. The problem of dependability arises because there are many kinds of nuclei in rat liver, and it is not known whether the various techniques used produce the same yield and distribution of nuclear types. This thesis will determine the effects, in respect to type and concentration, produced on the nuclei isolated by various conditions of homogenization of rat liver.

Liver tissue consists of several types of cells. There are parenchymal cells, sinusoid endothelial cells, connective tissue cells called stroma, bile duct cells, and the Kupffer phagocytic cells (Ham and Lesson, 1961). The literature suggests that the liver is about 60% parenchymal cells (Daoust, 1958). The parenchymal cells may possess one nucleus that has double or quadruple the volume of the basic nucleus; some have two or more small nuclei (Greep, 1966).

The works of Beams and King (1942) suggest that because of multiples of the usual diploid number of chromosomes, many of the liver cells are polyploid (Greep, 1966). Observation of stained and fixed sections show the variety of nuclei found in liver tissue. The parenchymal cells have nuclei that are ovoid to spherical (Ham, 1965). Kupffer cells

seem to have ovoid-shaped nuclei and the endothelial of the sinusoids display flattened dense nuclei; however, many of the nuclei of liver tissue are indistinguishable except the parenchymal nuclei (Greep, 1966).

Classes of parenchymal nuclei were established by Beams and King (1942) and Daoust and Cantero (1959) on the basis of diameters measured by ocular micrometry in stained and fixed sections of rat liver tissue. Three classes were determined. The nuclear classes have been shown to correspond to diploid, tetraploid, and octoploid cells with 1, 2, and 4 times the normal number of chromosomes or normal amount of deoxyribonucleic acid, respectively (Alfert, 1958).

Further classification has been done of nuclei isolated by homogenization and differential centrifugation. Falzone, Barrows, and Yiengst (1962) and Albrecht (1968) suggest three classes of liver nuclei, stromal (non-parenchymal), diploid, and tetraploid. They were able to define these three classes by the use of ocular micrometry based on the mean of three diameter ranges.

Falzone et. al. (1962) found that the largest percentage of the parenchymal nuclei in an adult rat are tetraploid and diploid, but that the octoploid and those of even higher ploidy were found in very small numbers.

With so many different kinds of nuclei in rat liver tissue it is possible that some of the methods of homogenization and centrifugation have not produced the best yield and

purity of isolated nuclei. Every technique of isolation of a cell component should attempt to satisfy the following conditions, preservation of its morphology and its biochemical composition, homogeneity of the fraction, and a sufficiently high yield (Chauveau, Moulé, and Rouiller, 1956).

Anderson (1955) suggests that since the homogenate may be considered a complex system, a number of factors may be expected to have marked effects on the kinds and condition of nuclei isolated.

Most investigators have used saline or sucrose solutions to prepare homogenates (Wilbur and Anderson, 1951).

Sucrose media are more efficient in separating nuclei from other cellular organelles such as mitochondria than saline media alone (Schneider, 1948). A sucrose medium alone without a salt causes clumping, distortion and contamination of nuclear preparations (Schneider and Peterman, 1950). An ion, usually divalent, is necessary to "harden" the nuclei without causing aggutination of the cytoplasm: $CaCl_2$ and $MgCl_2$ were found by Schneider and Peterman (1950) to be equally good for that purpose. Dauta-Mentré (1964) suggested the use of either Ca++ or K^+ in nuclear preparations to maintain the integrity of the nuclei. Hogeboom, Schneider, and Striebich (1952), Gill (1965), Allfrey, Littau, and Mirsky (1964), Chauveau et. al. (1956), and Anderson and Wilbur (1952) found that if $CaCl_2$ was used the result was a good yield of nuclei which were in excellent condition and the

preparations contain very few intact cells and mitochondria.

Dounce (1943a), Anderson (1955), and Dounce, Witter, Monty, Pate, and Cottone (1955) suggest preparing homogenates in a medium buffered at a near neutral pH (7.1 to 7.88), with the temperature maintained at 0° to 5° C during the process of homogenization.

Early investigators used differential centrifugation for nuclear isolation (Wilbur and Anderson, 1951).

Chauveau et. al. (1956) suggest that differential centrifugation, which is based on the sedimentation rates, allows contamination because all the particles migrate in the same direction, whereas density centrifugation enables a complete separation to be made because only particles having a density higher than that of the medium sediment out during centrifugation; the other particles migrate to the opposite direction. They also found that the yield was much better and the nuclei were completely free from cytoplasmic contamination and morphologically very well preserved. This was also supported by Gill (1965). Blobel and Potter (1966) reported that their techniques of homogenization and density centrifugation produced a great improvement of the purity and yield of nuclei.

Disruption of cells in preparing homogenates has been accomplished in many ways; by pressing the tissue through bolting silk after preliminary grinding, by use of tight fitting grinders or by use of a Waring Blender (Wilbur and

Anderson, 1951). It has been found that workers concerned with the technique of homogenization would prefer using the Dounce hand plunger or the Potter-Elvehjem motor driven Teflon pestle. These plungers work on the principles of fluid turbulence and shearing which has been reported to bring about better tissue disruption (Anderson, 1956).

With consideration given to the factors that may have marked effects on homogenate preparation and condition of isolated nuclei (such as pH, viscosity and composition of suspending media) (Anderson, 1955), and possibly the best method of homogenization and density centrifugation (Blobel and Potter, 1966), only varying the conditions of homogenization would have had profound effect on the distribution of the nuclei isolated from rat liver in this thesis project.

The purpose of this study was to show that by varying the conditions of homogenization for isolation of nuclei by the Blobel and Potter technique (1966), classes of nuclei could distinctly be determined and a condition of homogenization optimum for both purity and yield of nuclei could be established.

MATERIALS AND METHODS

All experiments were performed with albino rats (weights varying from 200 g to 300 g) of the Wistar strain. The rats were etherized and killed by decapitation. The livers were removed quickly, washed in tap water and minced with scissors, then chilled immediately in a volume (20 ml) of ice-cold 0.25 M sucrose in TKM (a buffered solution containing 0.05 M tris HCl, pH 7.5, at 20° C; 0.025 M KCl; 0.005 M $MgCl_2$). Liver volumes were also determined with a slight variation due to the sizes of the rats. The general methods of Blobel and Potter (1966) were used for the homogenate preparation and nuclear isolation of fresh rat liver, but some modifications were made.

A Dounce homogenizer tube and plungers of four different clearances were used to prepare four homogenates, each followed by a nuclear isolation in four separate experiments. All of these operations were carried out at 0° C.

The homogenizer's and plungers' diameters were determined by a micrometer and total clearances were calibrated between the homogenizer tube and plungers at room temperature and 0° C. The plungers were labeled as: T-1 (loosefitting Teflon; clearance at 0° C .0123 microns), T-2 (tight-fitting Teflon; clearance at 0° C .0095 microns), G-1 (loose-fitting Glass; clearance at 0° C .0041 microns), and G-2 (tight-fitting Glass; clearance at 0° C .0023 microns).

Preparations of all four major homogenates began with

the T-1 plunger for the first ten strokes, providing a condition of good tissue disruption and thereby being designated as zero strokes. The homogenate was strained through two layers of nylon mesh to remove connective tissue and a 5 ml sample was taken and prepared for nuclear isolation. The subsequent conditions of homogenization were done with the four plungers in five-stroke intervals up to thirty-five strokes in different experiments. Five ml samples were taken following each five-stroke interval and prepared for nuclear isolation, these were not strained. As a result this produced homogenate preparations at 0 (first 10 strokes), 5, 10, 15, 20, 25, 30, and 35 strokes, representing 32 conditions of homogenization for the four major homogenate preparations.

The nuclear isolation techniques were similar to Blobel and Potter (1966). The five ml samples were placed in eight centrifuged tubes of the Beckman Ultracentrifuge Model L3-40. Ten ml of 2.3 M sucrose (heavy sucrose) in TKM was added to each tube by means of a syringe and large needle and thoroughly mixed with the 0.25 M sucrose homogenate by inversion. The mixture was underlaid by 20 ml of heavy sucrose; the tip of the needle was placed at the bottom of the tube and the heavy sucrose was introduced slowly, forcing the lighter homogenate upward. The tubes were then spun in the Beckman Ultracentrifuge for 30 min at 40,000 rev/min in a Spinco 50.1 rotor (123,000 g, av.) at 0° to 4° C. After

centrifugation, tubes were removed and placed in an ice bath. The supernatant was suctioned off and materials adhering to the wall of the tubes were removed by a spatula, and the walls wiped dry with tissue. The nuclear pellet was taken up in 10 ml of 0.25 M sucrose in TKM. The tubes in an ice bath were placed in the refrigerator until analysis.

Nuclear analysis of each sample was done on the basis of nuclear numbers and sizes. Nuclear concentrations were determined with a hemocytometer (Falzone et. al., 1962). Three counts were made and the average of the counts were used. A few drops (three to five) of 1% methyl green were added to approximately one-half of the nuclear sample to facilitate observation of the nuclei while the counts were being made. Nuclear sizes were determined by ocular micrometer measurements of unstained nuclei to the nearest micrometer division (each division = 0.34 microns) on a Nikon phase contrast microscope with oil immersion. Diameters of 100 randomly chosen nuclei were measured from each of the samples. This was done for each of the eight samples differing in number of strokes for the four major preparations of rat liver homogenates, differing in plunger clearances.

Nuclear classes recognizable in the 3,200 measured nuclei were determined graphically. Nuclei of the same sizes from the 32 different homogenizations were lumped together and the totals of the various nuclear sizes were used to construct a graph. The low points following an apparent peak on

the graph were chosen as cut off points for the various classes. It was apparent that the nuclear diameter ranges were more varied and constituted more classes of nuclei than found in the literature (Falzone et. al., 1962; Albrecht, 1968). All further analysis grouped the nuclei into six classes on the basis of distribution.

A fifth experiment with the T-1 plunger was performed with the following stroke variation: 2-4-6-8-10 (unfiltered) -10 (unfiltered, equivalent to zero strokes for the other four major homogenate preparations)-5 (after zero), and 10 strokes. Five ml samples were taken after each varied stroke interval and prepared for nuclear isolation. Nuclear concentrations were determined and 100 diameters of randomly chosen nuclei of the eight samples were measured as in the other homogenate preparations.

The analysis of the data collected from the four major homogenate preparations was based on the number of strokes with each plunger as compared to the nuclei concentrations and percentages of nuclear classes. Statistically, this was done with the use of linear regression analysis. The t-test was employed to determine the significance of the data collected.

DATA AND DISCUSSION

Figure 1 shows graphically the diameters of all nuclei measured. The isolated nuclei could be divided into six classes based on diameters. The low points on the graph following the apparent peaks were used as cut off points. The larger nuclei (classes I, II, III, and IV) were assumed to be from parenchymal cells. These larger nuclei were in greater abundance, as would be expected from the literature (Daoust, 1958; Alfret, 1958; Falzone et. al., 1962; Albrecht, 1968). The elongated nuclei because of size (diameters greater than 10) could be parenchymal nuclei of higher ploidy. Classes II and IV (tetraploid and diploid) represented the largest percentages of nuclei, as would be expected in the liver of an adult rat (Falzone et. al., 1962).

Table 1 shows a representation of the nuclear class size ranges, the total of nuclei per nuclear class from the 32 homogenizations, and the percentages of the total number.

The counts and percentages from Tables 3 through 7 (see Appendix) show that the larger nuclei decrease with increasing number of strokes and the smaller nuclei increase somewhat with increasing stroke numbers. However, with a variation in number of strokes (0-35) the total nuclear count declined.

The data indicate that the larger nuclei were being broken up with increasing stroke numbers faster than they were released. Smaller nuclei definitely showed some increase

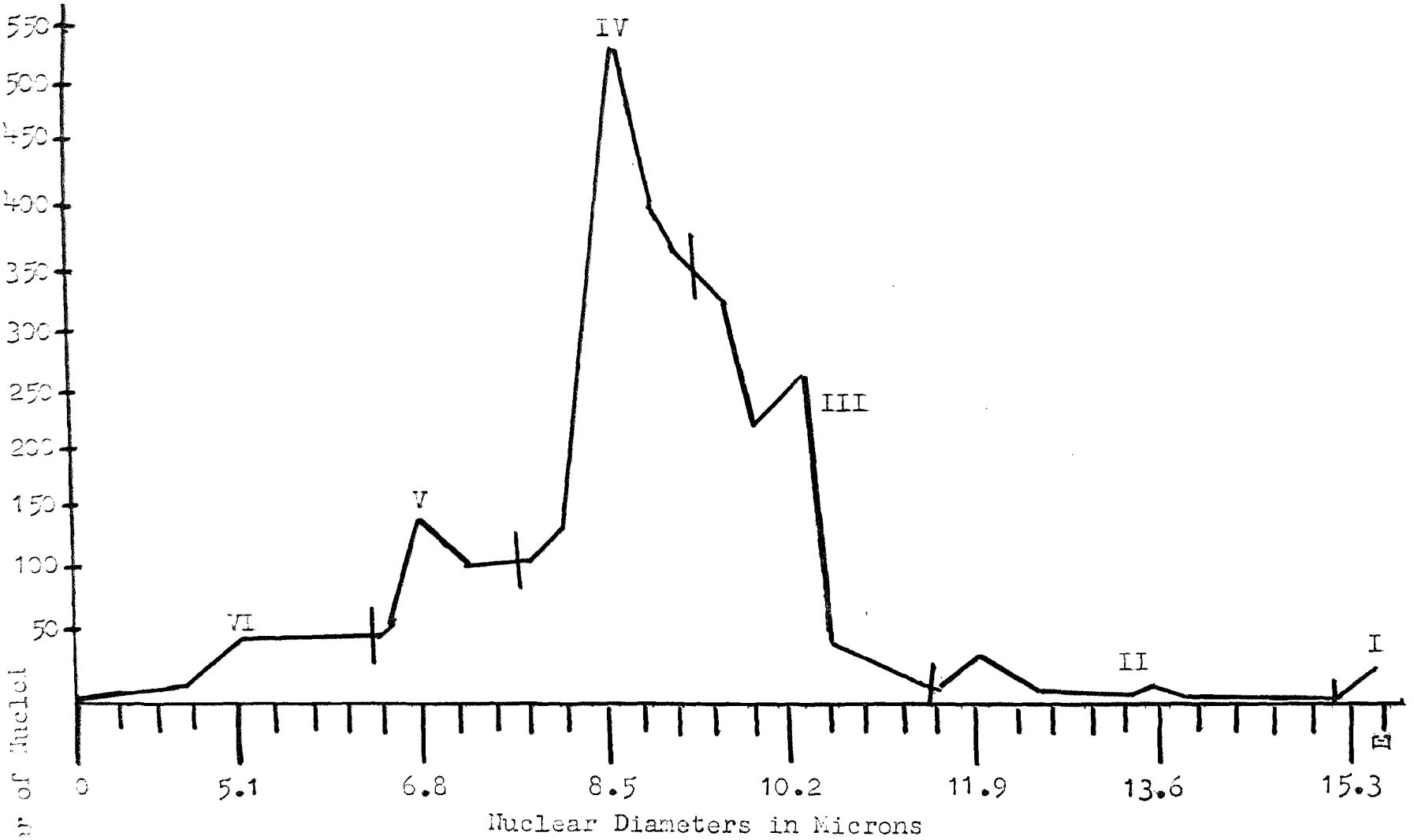

Figure 1. Distribution of measured nuclear diameters and establishment of nuclear classes. E-Elongated Nuclei.

Table 1. Nuclear diameter ranges, total numbers, and percentages for respective nuclear classes of the 3,200 measured isolated nuclei.

NUCLEAR CLASSES	NUCLEAR DIAMETER RANGES	TOTAL NUCLEAR NUMBERS PER CLASS	TOTAL NUCLEAR PERCENTAGES PER CLASS
I-Higher Ploidy	Elongated (diameters greater than 10 microns)	31	.97%
II-Octoploid	11.56 to 14.96 microns	66	2.06%
III-Tetraploid	9.86 to 11.22 microns	914	28.56%
IV-Diploid	7.82 to 9.52 microns	1590	49.69%
V	6.46 to 7.48 microns	412	12.88%
VI	4.08 to 612 microns	187	5.84%

in concentration with increasing number of strokes, they were released faster than they were destroyed. However, with the tighter plungers and the greater number of strokes, the numbers of non-parenchymal nuclei were constant or even decreased.

A formal analysis by linear regression (Table 2) support these conclusions from casual inspection of the data. From the t-test, correlation coefficients of .71 represents the condition of the data being statistically significant and .88 being highly significant. The values for class III nuclei (tetraploid) show a trend of decrease with increasing stroke numbers and a correlation coefficient significant for all four plungers. Class IV (diploid) shows a general trend in decrease and the correlation coefficient significant for plungers T-1 and G-1, and nearly a high significance for plunger T-1. The correlation coefficients for plungers T-2 and G-2 were high but not significant. Classes V and VI, the non-parenchymal nuclei, show a general trend of increase with increasing number of strokes, however, the correlation coefficients are low and not significant. For classes I and II, which could be of the higher ploidy parenchymal type nuclei, values were too low to be significant. However, class I does show high correlation for plungers T-1, T-2, and G-1 and class II for plunger T-2. Both classes show a general trend of decrease with increasing stroke numbers.

Figure 2 graphically shows that the best yield and

Table 2. Regression analysis of variations in number of each nuclear class with variation in number of strokes of homogenization. Symbols are $Y = mx + b$; where Y is number of nuclei; m is the slope; x is the number of strokes from 0 to 35; b is the Y intercept; r represents the correlation coefficient. (By the t-test with samples of eight pairs, a correlation coefficient of over .71 indicates that the slope m is significantly different from zero.) Clearances calibrated in microns.

PLUNGERS	T-1			T-2			G-1			G-2		
CLEARANCE	.0123			.0095			.0041			.0023		
LINEAR REGRESSION	r	b	m	r	b	m	r	b	m	r	b	m
CLASSES OF NUCLEI												
I	.31	7	2	.59	-112	31	.049	248	2	-.25	318	-7.4
II	-.48	.907	-15	-.60	1598	-.51	-.43	487	-9.5	-.25	365	-6.4
III	-.76	10296	-252	-.80	13368	-357	-.71	9539	-210	-.82	14036	-358
IV	-.87	13621	-196	-.57	12586	6113	-.72	13957	-161	-.57	12955	-136
V	.49	1516	36.6	.26	1920	25.8	-.25	4101	-16	-.23	4740	-71.7
VI	.25	1073	20	.12	76	6	.76	268	46	.20	1145	-7.4

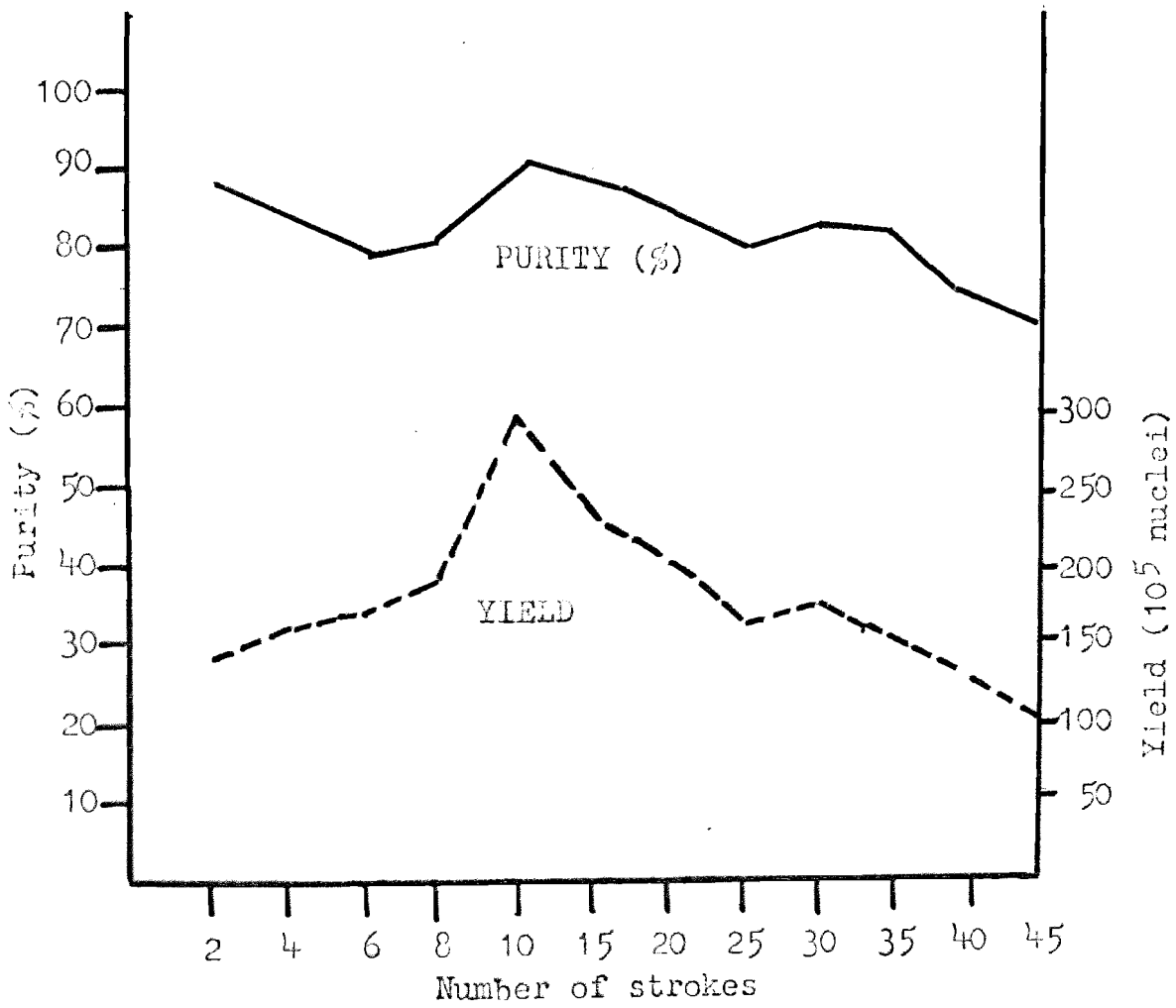

Figure 2. Combined data of all five experiments showing the average purity and yield of parenchymal nuclei from isolation using 2 to 45 strokes. (Note: the right side of the graph from 15 to 45 strokes is compressed).

purity of parenchymal nuclei would be from 8 to 15 strokes with 10 strokes being optimum. The graph shows that there is an increase in numbers of parenchymal nuclei with increasing stroke numbers up to 10 and then a general decline after ten strokes. The increasing number of strokes also had profound effects on the purity of nuclei isolated. The parenchymal nuclei showed an increase up to 10 strokes with a purity of 91% and a yield of 289 X 10^5 from a total count of 300 X 10^5. After a total of 45 strokes the purity of parenchymal nuclei was 72% and the yield 109 X 10^5. This was the general trend for purity and yield for plunger clearances (average), indicating that the most profound effects on the nuclei as a result of the 32 conditions of homogenization were brought about by the increase in stroke numbers and very little if any by the plunger clearances.

Tables 3 through 7 of the Appendix show the general decrease of nuclei after the condition of 10 strokes (zero strokes) in percentages and concentrations.

CONCLUSIONS

Isolated rat liver nuclei could be divided into six classes on the basis of diameters. Four of the six classes represented are the parenchymal type nuclei. The other two classes were non-parenchymal, probably sinusoids, bile duct, connective tissue, and Kupffer phagocytic cell nuclei.

The effects of conditions of homogenization can clearly be seen with increasing number of strokes and with little effect shown by the clearances of the four plungers.

Parenchymal nuclei predominated among the isolated nuclei under all conditions of homogenization. This is clearly indicated from the five varied homogenate preparations with an average of 91% parenchymal at the optimum 10 strokes and 72% parenchymal nuclei at 45 strokes.

The best combination of yield and purity of parenchymal nuclei, as well as ease of isolation was obtained with the Teflon plunger with a clearance of .0123 microns. Eight to fifteen strokes with this plunger produced a good yield and high purity of parenchymal nuclei with 10 strokes being optimum. The effects of homogenization after 10 strokes with increasing stroke numbers produced a decrease in concentration of almost all nuclear types, but especially of the parenchymal nuclei.

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APPENDIX

Table 3. Classes of nuclei in respect to total number, concentration per class, and per cent per class for each stroke-interval as a result of conditions of homogenization with plunger T-1. Total numbers and concentration = 10^5 nuclei.

STROKES (Plunger T-1; clearance = .0123).		0	5	10	15	20	25	30	35
TOTAL NUMBER OF NUCLEI		325	241.5	232.3	191.5	200	175	150	150
CLASSES OF NUCLEI									
I	Per Cent	0	0	0	1	0	0	0	1
	Concentration	0	0	0	1.9	0	0	0	1.9
II	Per Cent	3	5	2	1	3	4	6	1
	Concentration	9.8	12.1	4.7	1.9	6	7	9	1.5
III	Per Cent	47	33	26	21	20	33	22	16
	Concentration	152.8	62.8	60.4	40.2	40	57.8	33	24
IV	Per Cent	42	60	52	48	57	46	46	52
	Concentration	136.5	144.9	120.8	91.9	114	80.5	69	78
V	Per Cent	7	2	7	17	11	9	20	19
	Concentration	22.8	4.8	16.3	32.6	22	15.8	30	28.5
VI	Per Cent	1	0	13	12	9	8	6	11
	Concentration	3.3	0	30.2	23	18	14	9	16.5

Table 4. Classes of nuclei in respect to total number, concentration per class, and per cent per class for each stroke-interval as a result of conditions of homogenization with plunger T-2. Total numbers and concentration = 10^5 nuclei.

STROKES (Plunger T-2; clearance = .0095)		0	5	10	15	20	25	30	35
TOTAL NUMBER OF NUCLEI		325	275	250	216.5	190	175	166.5	150
CLASSES OF NUCLEI									
I	Per Cent	1	1	0	0	0	0	1	8
	Concentration	3.3	2.8	0	0	0	0	1.7	12
II	Per Cent	9	2	3	3	0	0	0	3
	Concentration	29.3	5.5	7.5	6.5	0	0	0	4.5
III	Per Cent	56	26	45	17	43	18	13	21
	Concentration	182	71.5	112.5	36.8	81.7	31.5	21.6	31.5
IV	Per Cent	32	49	43	60	47	69	62	39
	Concentration	104	134.8	107.5	129.9	89.3	120.8	103.2	58.5
V	Per Cent	2	15	6	15	7	11	20	19
	Concentration	6.5	41.3	1.5	32.5	13.3	19.3	33.3	28.5
VI	Per Cent	0	7	3	5	3	2	4	10
	Concentration	0	19.3	7.5	10.8	5.7	3.5	6.7	15

Table 5. Classes of nuclei in respect to total number, concentration per class, and per cent per class for each stroke-interval as a result of conditions of homogenization with plunger G-1. Total numbers and concentrations = 10^5 nuclei.

STROKES (Plunger G-1; clearance = .0041)		0	5	10	15	20	25	30	35
TOTAL NUMBER OF NUCLEI		325	250	233.4	220	207.5	200	190	175
CLASSES OF NUCLEI									
I	Per Cent	0	0	0	8	0	1	1	1
	Concentration	0	0	0	17.6	0	2	1.9	1.8
II	Per Cent	2	2	0	3	0	1	2	1
	Concentration	6.5	5	0	6.6	0	2	3.8	1.8
III	Per Cent	40	21	42	19	16	14	24	12
	Concentration	130	52.5	98	41.8	33.2	28	45.6	40.3
IV	Per Cent	45	55	41	47	66	58	38	47
	Concentration	146.3	137.5	95.7	103.4	137	116	72.2	82.5
V	Per Cent	13	16	16	19	14	19	27	15
	Concentration	42.3	40	37.3	41.8	29.1	38	51.3	26.3
VI	Per Cent	0	6	1	4	4	7	8	13
	Concentration	0	15	2.3	8.8	8.3	14	15.2	22.8

Table 6. Classes of nuclei in respect to total number, concentration per class, and per cent per class for each stroke-interval as a result of conditions of homogenization with plunger G-2. Total numbers and concentrations = 10^5 nuclei.

STROKES (Plunger G-2; clearance = .0023)		0	5	10	15	20	25	30	35
TOTAL NUMBER OF NUCLEI		325	290	240	225	207.5	200	117	133
CLASSES OF NUCLEI									
I	Per Cent	3	0	0	0	0	0	0	4
	Concentration	9.8	0	0	0	0	0	0	5.3
II	Per Cent	1	0	4	0	1	1	2	0
	Concentration	3.3	0	9.6	0	2.1	2	3.3	0
III	Per Cent	59	38	27	37	15	34	23	26
	Concentration	191.8	110.2	64.8	83.3	31.1	68	38.3	34.5
IV	Per Cent	28	51	53	44	65	49	54	44
	Concentration	91	147.9	127.2	99	134.9	98	89.9	58.3
V	Per Cent	6	6	10	15	11	12	15	12
	Concentration	19.5	17.4	24	27	22.8	24	25	15.9
VI	Per Cent	3	5	6	4	8	4	6	14
	Concentration	9.8	14.5	14.4	9	16.6	8	10	18.5

Table 7. Classes of nuclei in respect to total number, concentration per class, and per cent per class for each stroke-interval as a result of conditions of homogenization with Plunger T-1 beginning with 2 strokes. Total numbers and concentration = 10^5 nuclei.

STROKES (Plunger T-1; clearance = .0123)		2	4	6	8	10 unfiltered	10 filtered	5	10
TOTAL NUMBER OF NUCLEI		166.5	191.5	200	233.3	266.5	307.4	275	300
CLASSES OF NUCLEI									
I	Per Cent	1	1	0	0	2	0	0	0
	Concentration	1.7	1.9	0	0	5.3	0	0	0
II	Per Cent	4	1	1	0	2	1	1	5
	Concentration	6.7	1.9	2	0	5.3	3.1	2.8	15
III	Per Cent	39	35	34	44	38	49	29	43
	Concentration	64.9	67	68	102.6	101.2	150.6	79.8	129
IV	Per Cent	44	46	44	37	50	33	52	45
	Concentration	73.3	88.1	88	86.3	133.1	101.4	143	135
V	Per Cent	12	16	21	18	7	17	18	5
	Concentration	20	20.6	42	42	18.6	52.2	49.5	15
VI	Per Cent	0	1	0	1	1	0	0	2
	Concentration	0	1.9	0	2.3	2.7	0	0	6